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19. ABSTRACT (Continue on reverse if necessary and identify by block number)

The goal of this project is to design and synthesize totally new, man-made enzymes. A peptide structure having catalytic activity resembling that of chymotrypsin was designed by computer graphics modelling and synthesized by solid phase peptide synthesis methods. This synthetic peptide, having 72 amino acid residues, has activity to catalyze the hydrolysis of small molecule substrates for chymotrypsin.

The initial goal of this project was thus accomplished.

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FINAL REPORT August 1989

ONR CONTRACT N00014-86-K-0476; Project 441g015

PRINCIPAL INVESTIGATOR: John M. Stewart

CONTRACTOR: Univ. of Colorado Health Sciences Center

CONTRACT TITLE: Synthetic Helizyme Enzymes

START DATE: 1 August 1986; END DATE: 31 July 1989

RESEARCH OBJECTIVE: The goal of this project was to design, synthesize and test totally new, synthetic peptides with enzyme activity. That is, to design and synthesize a man-made enzyme. The ultimate long-term goal was to synthesize a molecule with acetylcholine esterase activity. As a model along the way, a molecule with chymotrypsin activity was the initial goal.

PROGRESS: Good progress has been made. We have accomplished the first ever synthesis of a man-made enzyme. We have designed and synthesized a peptide having catalytic activity resembling that of alpha-chymotrypsin.

Using the Silicon Graphics IRIS computer (purchased principally with ONR funds) and the SYBYL-MENDYL software from Tripos Assoc., we have loaded a composite alpha-chymotrypsin structure from the Brookhaven data base of X-ray structures. Maintaining the three-dimensional positions of the active site residues, the remainder of the supporting protein structure was removed. Using our knowledge of the forces which govern folding of peptide chains into alpha-helices, we designed a four-chain bundle of helical peptides to hold the active site residues in exactly the positions they occupy in chymotrypsin.

Since these new enzymes are designed to be fully helical, we call them generically "helizymes." Our first design, having chymotrypsin activity we call "chymohelizyme #1" (abbreviated CHZ1).

Basic design principles are:

1. Four amphipathic helices adhere with their hydrophobic surfaces facing the interior of the assembly. Principles learned from naturally-occurring "leucine zipper" segments of natural proteins were employed to confer added stability to the assembly.

2. Carboxy- (C-) terminals of the helical chains are held together in the right conformation by utilizing the side-chain functional groups of lysine and ornithine residues.

3. The C-terminus is synthesized as an amide, and all amino- (N-) termini are acetylated to promote helix formation.

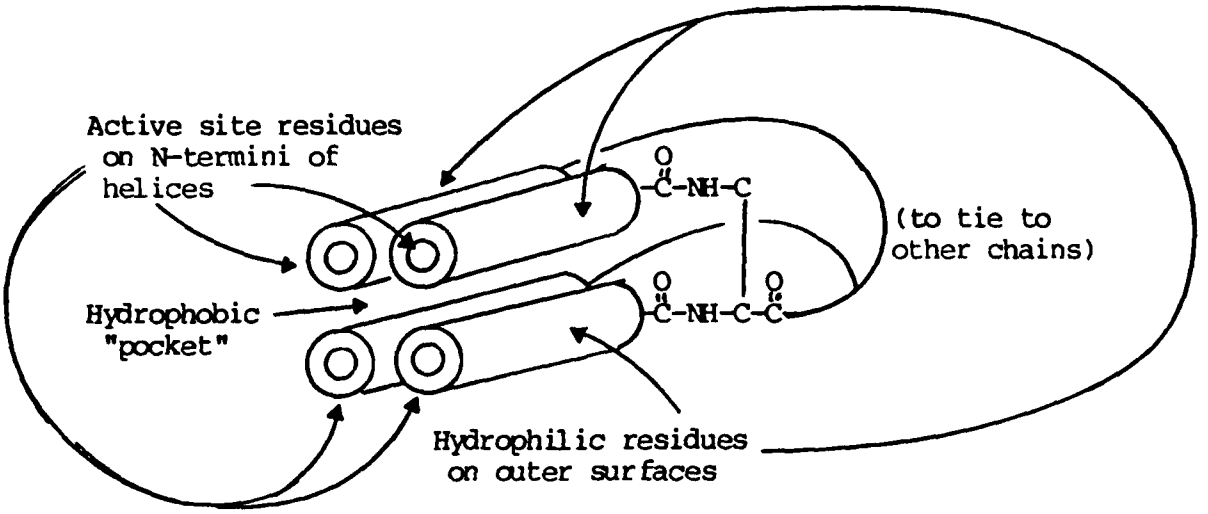
4. The active site Ser, His and Asp residues are situated at the N-terminus of three different chains. This location is fully compatible with the propensity of these residues to occur in proteins at the N-terminus of helical regions.

5. The essential "oxyanion hole" of the active site is constituted by parts of the fourth chain.

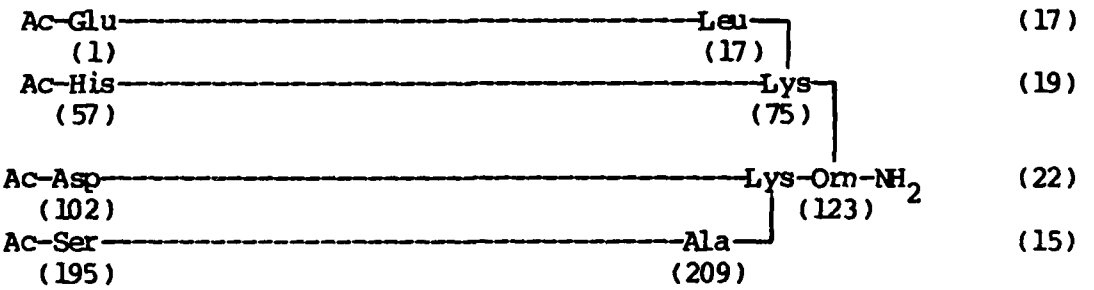
6. Glu and Lys residues are placed at strategic locations on the exterior of the helices to provide inter-turn ionic bonds within each helix and inter-helix bonds between contact points of adjacent helices.

7. Energy minimization was used repeatedly to assure that the final structure was thermodynamically preferred and stable.

The overall structure of the assembled peptide resembles this diagram:




The general chemical structure of Chymohelizyme #1 is shown in the following diagram. Three of the chains begin with the residues of the chymotrypsin catalytic triad, and the residues are numbered accordingly (His57, Asp102 and Ser195 in chymotrypsin), while the fourth chain begins with a glutamic acid residue, arbitrarily numbered 1. This fourth chain provides the "oxyanion hole" necessary for the catalytic action of the enzyme. All N-termini are acetylated, to promote helix formation. Residues along the chains are then numbered in sequence from these residues; these sequence numbers are given in parentheses. The numbers beyond the C-terminal ends of the chains in the diagram give the number of residues in that chain (17, 19, 22 and 15 residues, respectively). The single C-terminal residue (Orn 123) is blocked with the amide function, to promote helix formation, although this can be modified to provide linkage to an insoluble support via a spacer for construction of immobilized enzymes, for example.




Computer-generated stereoscopic side view and end view drawings of this structure are given on the next page. (These are relaxed stereo drawings and can be viewed with standard stereo viewers or directly by those persons who have learned to relax their eyes). The N-terminal and C-terminal residues of all chains are labeled for identity, but other residues are not labeled, to prevent clutter in the drawing. The substrate used in the design work, acetyl tyrosine ethyl ester (ATEE) is a standard much-used chymotrypsin substrate. It is shown docked in the active

site. The backbones of the four helices constituting the main framework of the structure are shown in narrow lines. These backbones can be picked out in the side view, but show dramatically in the end view, where the viewer is looking directly down the axes of the helices.

SYNTHESIS OF CHYMOHELIZYME #1

The entire 4-chain amino acid sequence was assembled in one operation by SPPS, using MBHA resin. Boc, Fmoc and Npys alpha-protecting groups were used in various parts of the synthesis, as needed for selectivity. Benzyl-related side chain blocking groups were used (except in assembly of the C-terminal linker structure of Lys and Orn); these were removed simultaneously with cleavage of the peptide from the resin by HF. Coupling reactions were monitored at nearly all steps using qualitative and quantitative ninhydrin reactions. Quantitative coupling at each step was assured by use of the most sophisticated chemistry available. Aliquots of peptide-resin were removed at appropriate intervals for HF cleavage, hydrolysis and amino acid analysis.

Initial purification of the final crude peptide was by chromatography on a calibrated Sephadex G-50 column in 30% acetic acid. In addition to non-peptide and small molecules, the product consisted chiefly of two peaks, consistent with monomer and dimer. Dimerization was very persistent, and probably represents an extended, flat array in which the hydrophobic sides of two "sheet" arrays of helices interact. Aqueous solutions of monomer slowly associate with concomitant loss of catalytic activity. We also found that the product has an extremely high affinity for anisole, which was used as part of the scavenger mix used in the HF cleavage of the peptide-resin. Unfortunately, anisole is a specific inhibitor of chymotrypsin. The high affinity of our peptide for anisole attests to the successful design of a hydrophobic binding pocket. Amino acid analysis of the product shows the correct composition.

The semi-purified peptide shows significant helix in neutral aqueous solution, as determined by molar ellipticity at 222nm in circular dichroism (CD) spectroscopy. In 80% ethanol solution, CD indicated presence of at least 80% helix, nearly the maximum amount possible for this structure.

Assay of the semi-purified chymohelizyme against the chymotrypsin substrates benzyloxycarbonyl-L-tyrosine p-nitrophenyl ester (ZTONP) and benzoyl L-tyrosine ethyl ester (BTEE) by standard methods shows that our product has significant, but low, catalytic activity. Hydrolysis is catalytic; that is, more than one mole of substrate was hydrolyzed per mole of enzyme. Hydrolysis of BTEE showed an activity approximately 0.1% that of Chymotrypsin in the same system. Maximum catalytic rate was seen at pH 7.82; there was still catalysis at slightly higher and lower pH, but none at pH 6 or 8.5. There is no spontaneous hydrolysis of BTEE in this system.

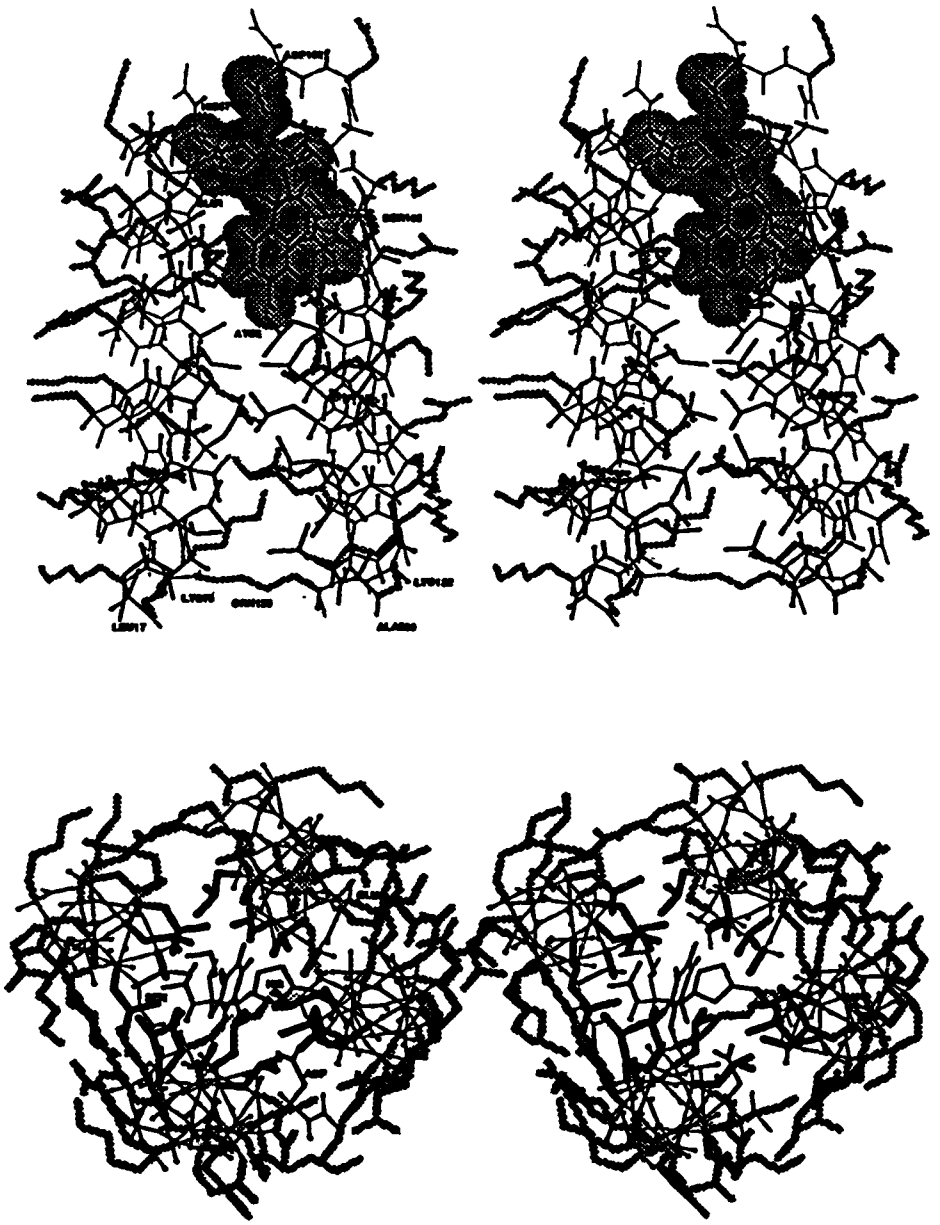
Since the amino acid residues which constitute the active site in Chymohelizyme #1 are all located on different peptide chains, the fact that we have enzyme activity validates our design parameters and synthetic methodology.

INVENTIONS: We have invented the first man-designed enzyme. We are proceeding to secure protection for this invention.

PUBLICATIONS: None yet. We will publish as soon as our studies of the enzyme activity of CHZ1 are complete.

TRAINING: Wieslaw A. Klis, citizen of Poland, synthesized CHZ1.

AWARDS: None.



Relaxed stereo drawings of "Chymohelizyme #1" structure. Internal hydrophobic side chains are dark and heavy; external polar side chains are broad, light and stippled. Helix backbone chains and N-terminal acetyl groups are in narrow lines. Hydrogen atoms are shown only on alpha carbons, backbone nitrogens and the substrate; backbone hydrogens are shown as small "knobs" to aid in following the helix. The substrate, acetyl tyrosine ethyl ester (ATEE), is docked in the active site of the enzyme. TOP- Side view, with Glu1 and Ser195 chains in the foreground. N-terminals are at the top; C-terminals at the bottom. Amino acid residues at the ends of the chains are labeled. Van der Waals shells are added to the substrate and the catalytic triad residues in the enzyme. BOTTOM- End view, with the N-terminal catalytic site in the foreground. N-terminal residues are labeled. The aromatic ring of the substrate appears just to the left of the ring of the catalytic histidine in the enzyme.

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